Mitochondrion 13 (2013) 835–840

Contents lists available at ScienceDirect

Mitochondrion

journal homepage: www.elsevier.com/locate/mito

Mitochondrial response to oxidative and nitrosative stress in early stages of diabetes

Ruth Noriega-Cisneros [a], Christian Cortés-Rojo [a], Salvador Manzo-Avalos [a], Mónica Clemente-Guerrero [a], Elizabeth Calderón-Cortés [b], Rafael Salgado-Garciglia [a], Rocío Montoya-Pérez [a], Istvan Boldogh [c], Alfredo Saavedra-Molina [a,c,*]

[a] Instituto de Investigaciones Químico-Biológicas, Universidad Michoacana de San Nicolás de Hidalgo, Morelia, Mich., Mexico
[b] Facultad de Enfermería, Universidad Michoacana de San Nicolás de Hidalgo, Morelia, Mich., Mexico
[c] University of Texas Medical Branch at Galveston Texas, Galveston, TX, USA

ARTICLE INFO

Article history:
Received 13 July 2012
Received in revised form 4 May 2013
Accepted 30 May 2013
Available online 7 June 2013

Keywords:
Diabetes
Oxidative stress
Nitrosative stress
Brain mitochondria
Glutathione
Antioxidant

ABSTRACT

Diabetes mellitus (DM) is associated with increased production of reactive oxygen and nitrogen species; consequently, an increase in lipid peroxidation and a decrease in antioxidants resulting in mitochondrial dysfunction. Using a rat model of DM induced by streptozotocin, we show the opposite: an increase in NO levels, S-nitrosylation, aconitase activity, and total glutathione and a decrease in lipid peroxidation at early stages of diabetes. These data imply that the decrease in lipid peroxidation is a vital early response to hyperglycemia to prevent escalation of ROS generation in mitochondria. These results also suggest a need for novel therapeutic targets to prevent the neurological consequences of diabetes.

1. Introduction

Diabetes is a metabolic disorder of multiple etiologies, characterized by chronic hyperglycemia caused by defects in the secretion of insulin or the action of insulin, or both. Symptoms of diabetes mellitus (DM) are not severe or may be absent in the early stages of the disease and consequently, hyperglycemia may be present and cause pathological and functional changes before a diagnosis is made (NDDG, 1979; WHO, 1980). The brain is one of the organs in which diabetes causes electrophysiological and degenerative changes and a decrease in cognitive functions, which collectively are termed diabetic encephalopathy (Biessels et al., 1994; Manschot et al., 2007; Mastrocola et al., 2005; Reske-Nielsen et al., 1965; Stewart and Liolitsa, 1999). Also, the damage in different brain structures as cortical and hippocampus become exacerbated by initial recurrent stages of hyperglycemia (Cardoso et al., 2010, 2013), as well as in retina (Baptista et al., 2011). The development of diabetic encephalopathy is a multifactorial process in which both vascular and metabolic factors are involved, including chronically elevated intracellular glucose concentrations (Mastrocola et al., 2005).

The direct toxicity of glucose in neurons is due to increased production of reactive oxygen species (ROS) by NADPH oxidases and the mitochondrial electron transport chain (ETC) (Rice-Evans and Burdon, 1993). The mitochondrial inner membrane is a primary target of ROS resulting in oxidation of polyunsaturated fatty acids (Rice-Evans and Burdon, 1993). Lipid peroxidation, along with oxidative modifications of ETC proteins, leads to changes in electron flow and electron leakage, causing further ROS generation and additional damage to the mitochondrial ETC. Thus, mitochondrial dysfunction is considered a hallmark of DM, as has been shown in the hippocampus of diabetic rats (Reagan et al., 1999). Another example showed significant decreases of glycolytic enzymes and in the tricarboxylic acid cycle, the alpha-ketoglutarate dehydrogenase that were described in intracerebroventricular application of STZ (Hoyer and Lannert, 2008).

In mitochondria, nitric oxide (NO) is synthesized by the mitochondrial isoform of nitric oxide synthase (mtNOS) (Beckman, 1996). Mitochondrial superoxide ($O_2^{\bullet-}$) may react with nitric oxide (NO) to form peroxynitrite ($ONOO^-$). In turn, due to its reactivity, $ONOO^-$ can induce both oxidative and nitrosative reactions such as the nitration of protein-bound tyrosine residues to generate 3-nitrotyrosine (Beckman, 1996).

Mastrocola et al. (2005) has reported that hyperglycemia induced by STZ augmented oxidative stress in rat brain mitochondria and decreased both the expression and the activity of superoxide dismutase (SOD). These changes were parallel with increased expression and activity of mtNOS. Therefore, it was hypothesized that ETC complexes

* Corresponding author at: Instituto de Investigaciones Químico-Biológicas, Universidad Michoacana de San Nicolás de Hidalgo, Edificio B-3. C.U, Morelia, Mich. 58030, Mexico. Tel.: +52 443 326 5790x117; fax: +52 443 326 5788.
E-mail address: saavedra@umich.mx (A. Saavedra-Molina).

1567-7249/$ – see front matter © 2013 Elsevier B.V. and Mitochondria Research Society. All rights reserved.
http://dx.doi.org/10.1016/j.mito.2013.05.012

could also be impaired by nitrosative stress; however, no experimental evidence of increased nitrosative stress was shown. Therefore, to have more insight into the complex interplay between the factors that increase oxidative and nitrosative stress in brain mitochondria during diabetes, we decided to explore the effect of the early stages of diabetes in mitochondrial lipoperoxidation, nitrosative stress, total glutathione, and aconitase activity. We discuss the results obtained from these experiments considering the relations that exist between aconitase levels, glutathione synthesis, and lipoperoxidation to emphasize the importance of the alterations of those parameters during the choice or the design of an antioxidant to cope with brain mitochondrial dysfunction during diabetes.

2. Materials and methods

2.1. Animals

Male Wistar rats weighing 350–450 g were fed a "pellet" diet and water ad libitum (except in the fasting period). Hyperglycemia was induced by a single intraperitoneal injection of STZ (40 mg/kg) after a 12 h fasting period. STZ solution should be prepared just before use, since it is unstable, in citrate buffer pH 4.5 due to optimum solution stability achieved at this pH (Burcelin et al., 1993). The rats were euthanized at weeks 1, 3, and 5 after STZ/vehicle administration. Their weights were measured and glucose concentration was determined in blood using a commercial glucometer (Accu Check Sensor III, Bayer). Only STZ-administered rats with blood glucose levels above 300 mg/dL were regarded as diabetic. All animal procedures were carried out in compliance with Federal Regulations for the Use and Care of Animals (NOM-062-ZOO-1999, Ministry of Agriculture, Mexico), and were approved by the Institutional Committee of the *Universidad Michoacana de San Nicolás de Hidalgo*, for the Use and Care of Animals.

2.2. Isolation of mitochondria

Mitochondria were isolated by differential centrifugation in a Percoll gradient as previously described (Sims, 1990; Thakar and Hassan, 1988). Briefly, the entire brain, minus the cerebellum, was extracted and placed in a cold medium containing 210 mM mannitol, 70 mM sucrose, 1 mM EGTA, 0.5% bovine serum albumin, and10 mM MOPS (pH 7.4). The brain was homogenized manually in a glass homogenizer and centrifuged at 400 ×*g* and the obtained supernatant was centrifuged at 9000 ×*g*. Centrifugations were carried out for 10 min at 4 °C. The pellet obtained was suspended in 15% Percoll and placed in a discontinuous gradient of Percoll (23% and 40%). The gradient was centrifuged for 6 min at 30,700 ×*g* and band 3 was extracted, diluted at 1:4, centrifuged at 16,700 ×*g*, and washed in isolation medium supplemented with 0.5% bovine serum albumin, followed by centrifugation at 6900 ×*g* for 10 min. Mitochondrial protein concentration was measured by a modification of the Lowry method calibrated with bovine serum albumin (Lowry et al., 1951).

2.3. Assessment of L-citrulline levels

L-Citrulline production was determined as previously described (Knipp and Vasák, 2000), in a medium containing 190 mM mannitol, 5 mM KH_2PO_4, 15 mM KCl, 3 mM $MgCl_2$, 1 mM EGTA, and 10 mM MOPS (pH 7.4), plus 10 mM succinate and 2 μM rotenone. Mitochondria were incubated 1 h at 30 °C, in a shaking bath (30 rpm). L-citrulline was quantified in a Perkin-Elmer Lambda 18 UV/VIS spectrophotometer at 530 nm using a calibration curve.

2.4. Determination of mitochondrial lipid peroxidation

Mitochondrial lipid peroxidation (LPO) was determined by the thiobarbituric acid (TBA) assay as described by Buege and Aust (1978). Brain mitochondria (0.15 mg) were suspended in phosphate buffer (0.15 M) and mixed with 2 mL of an acid solution containing 15% trichloroacetic acid, 0.375% thiobarbituric acid, and 0.25 N HCl. The mixture was heated in boiling water for 15 min, ice-cooled, and centrifuged at 4410 ×*g* for 5 min. Absorbance of the supernatant was measured at 532 nm with a Perkin Elmer Lambda 18 UV/VIS spectrophotometer. Data were expressed as nanomoles of TBA reactive species (TBARS)/mg protein and were calculated employing the malondialdehyde molar extinction coefficient of $1.56 \times 10^5\ M^{-1}\ cm^{-1}$.

2.5. Determination of mitochondrial total glutathione

The measurement of total glutathione (GSH + GSSH) in mitochondria was conducted by spectrophotometric analysis of DTNB (5′,5-dithiobis-2-nitrobenzoic acid) reduction as described previously by Sies and Akerboom (1984), following the manufacturer's specifications (Sigma-Aldrich). DTNB reacts with GSH to form a product with absorption maximum at 412 nm. A calibration curve with GSH was performed for each experiment. Measurements were carried out on an ELISA microplate reader (Bio Rad Benchmark).

2.6. Assessment of aconitase activity

Aconitase activity was evaluated using citrate as the substrate and coupling the reaction to $NADP^+$-isocitrate dehydrogenase in a medium containing 27 mM Tris (pH 7.4), 5 mM citrate, 0.2 mM $NADP^+$, 0.6 mM $MnCl_2$, 1 unit $NADP^+$-dependent isocitrate dehydrogenase, and 50 μg of protein (Drapier and Hibbs, 1996; Rose and O'Connell, 1967). Mitochondrial pellets used for aconitase assays were vortexed for 5 min after resuspension in a small volume of 50 mM Tris (pH 7.4), 0.02% (v/v) Triton X-100 and centrifuged at 8000 ×*g* for 5 min at 4 °C. Aconitase activity was measured spectrophotometrically by recording the change in absorbance at 340 nm and using the NADPH molar extinction coefficient of $6.22\ mM^{-1}\ cm^{-1}$.

2.7. Assessment of protein nitrosylation

Samples containing 40 μg protein were resolved on 10% SDS-PAGE, and then blotted on PVDF membranes (Amersham Biosciences). The membranes were blocked with 1% TBS-T-gelatin for 1 h at room temperature and then incubated with an anti-3-nitrotyrosine antibody (anti-3-NT, Molecular Probes) diluted at 1:2000. The membranes were incubated with peroxidase-labeled secondary antibody (1:4000; goat anti-rabbit, Southern Biotech) prepared in 1% TBS-T-gelatin. Immunoreactive proteins were detected with a chemiluminescence assay (Western Blotting Luminol Reagent, Santa Cruz Biotechnology). Specific bands were quantified by densitometry using ImageJ software v. 1.46h.

2.8. Statistics

Data are presented as the mean ± SEM of at least 3 measurements from different animals. Statistical significance of the data was determined with the Student's *t* test using Prisma Plot software v. 5.0. Differences were considered statistically significant when $p < 0.05$.

3. Results

3.1. Glycemia and weight in control and diabetic rats

Weight and glucose levels were evaluated to assess progression of DM (Fig. 1). The weight of the control animals was constant throughout the experiment, while this parameter was significantly decreased ($p < 0.01$) (Fig. 1A) in the diabetic animals. Glucose levels in the control group were about 100 mg/dL. In the diabetic animals the glucose levels